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NLRP1 promotes tumor growth by enhancing inflammasome activation and suppressing apoptosis in metastatic melanoma

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Abstract

Inflammasomes are mediators of inflammation, and constitutively activated NLRP3 inflammasomes have been linked to IL-1β-mediated tumorigenesis in human melanoma. Whereas NLRP3 regulation of caspase-1 activation requires the adaptor protein ASC, caspase-1 activation by another danger-signaling sensor NLRP1 does not require ASC because NLRP1 contains a C-terminal CARD domain that facilitates direct caspase-1 activation via CARD-CARD interaction. We hypothesized that NLRP1 has additional biological activities besides IL-1β maturation and investigated its role in melanoma tumorigenesis. *NLRP1* expression in melanoma was confirmed by analysis of 216 melanoma tumors and 13 human melanoma cell lines. Unlike monocytic THP-1 cells with prominent nuclear localization of NLRP1, melanoma cells expressed NLRP1 mainly in the cytoplasm. Knocking down NLRP1 revealed a tumor promoting property of NLRP1 both *in vitro* and *in vivo*. Mechanistic studies showed that caspase-1 activity, IL-1β production, IL-1β secretion, and NF-kB activity were reduced by knocking down of NLRP1 in human metastatic melanoma cell lines 1205Lu and HS294T, indicating that NLRP1 inflammasomes are active in metastatic melanoma. However, unlike previous reports showing that NLRP1 enhances pyroptosis in macrophages, NLRP1 in melanoma behaved differently in the context of cell death. Knocking down NLRP1 increased caspase-2, -9, and -3/7 activities and promoted apoptosis in human melanoma cells. Immunoprecipitation revealed interaction of NLRP1 with CARD-containing caspase-2 and -9, whereas NLRP3 lacking a CARD motif did not interact with the caspases. Consistent with these findings, NLRP1 activation but not NLRP3 activation reduced caspase-2, -9,

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY INFORMATION

Supplementary Information accompanies the paper on the *Oncogene* website (http://www.nature.com/onc).

and -3/7 activities and provided protection against apoptosis in human melanoma cells, suggesting a suppressive role of NLRP1 in caspase-3/7 activation and apoptosis via interaction with caspase-2 and -9. In summary, we showed that NLRP1 promotes melanoma growth by enhancing inflammasome activation and suppressing apoptotic pathways. Our study demonstrates a tumor-promoting role of NLRP1 in cancer cells.

Keywords

melanoma; NLRP1; inflammasome; apoptosis; tumor

INTRODUCTION

Inflammation has long been recognized to be closely associated with various types of cancer.[1–3] Inflammation was initially believed to be a host defense mechanism against tumors; however, growing evidence suggests that tumor-derived inflammatory factors are required for tumor development and progression.[4–7] In particular, a pleiotropic cytokine IL-1β is secreted by human melanoma and promotes tumor growth, angiogenesis, and metastasis in both autocrine and paracrine manners.[6–8] The processing of IL-1β precursor depends on cytosolic caspase-1 activation, which is tightly regulated by NLRP (NACHT, LRR and PYD domains-containing protein) inflammasomes, including NLRP1 and NLRP3 inflammasomes.[9,10] During malignant transformation, inflammasomes are activated by danger signals arising from the tumor microenvironment.[9,10] We have reported that NLRP3 inflammasomes are constitutively activated and mediate autoinflammation via caspase-1 processing and IL-1β secretion in human melanoma cells.[8] NLRP3 regulation of caspase-1 activation requires the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain)). However, low expression of ASC in metastatic melanoma cells promotes tumor growth, but on the other hand, high expression of ASC in primary melanoma cells suppresses tumor growth,[11] suggesting that other NLRPs besides NLRP3 may contribute to the regulation of melanoma-related inflammation.

NLRP1 is unique in that unlike other NLRPs, it contains a CARD binding motif at its C terminus, which facilitates protein binding through CARD-CARD interactions. [9,10,12] Caspase-1 activation by NLRP1 therefore does not strictly require ASC.[9,10,12] In addition, CARD domains are typically found in proteins involved in caspase processing and activation, including caspase-1, -2, -5, and -9.[13,14] Caspase-1 and -5 are inflammatory caspases involved in the inflammasome activation responsible for IL-1β processing, whereas caspase-2 and -9 are apoptotic caspases involved in the mitochondrial apoptotic pathway.[9,10] We hypothesized that the CARD domain of NLRP1 may allow this danger-signaling sensor protein to play a distinct biological role in tumorigenesis.

Our findings demonstrate that NLRP1 promotes tumorigenesis in human melanoma. We provide evidence that NLRP1 coordinately enhances caspase-1-mediated inflammasome activation and suppresses the caspase-2/9-mediated mitochondrial apoptotic pathway in metastatic melanoma. Accordingly, NLRP1 may be a potential novel therapeutic target in human melanoma.

RESULTS

NLRP1 is expressed in human melanoma cells

NLRP1 is widely expressed in many human tissues.[15–17] We examined *NLRP1* RNA expression in human melanoma tissues using three publicly available microarray data sets from independent gene profiling studies.[18–20] The skin has the highest expression level of *NLRP1* RNA of all human tissues,[17] and was used as a positive control. These data showed that, *NLRP1* RNA is expressed in human melanoma tissues, though melanoma tissues have lower *NLRP1* RNA expression than normal skin (Figure 1a). Two of the three studies showed no difference in levels of *NLRP1* RNA between primary melanoma and metastatic melanoma, while one study reported a reduction in *NLRP1* RNA in human metastatic melanoma.

We then evaluated the expression of *NLRP1* RNA in 13 human melanoma cell lines derived from different stages of disease progression. Human monocytic THP-1 cells were used as a positive control because this cell line expresses NLRP1 and NLRP3, and has been studied for inflammasome functions and activation mechanisms.[8,16,21–23] *NLRP1* RNA was expressed in all melanoma cells tested, including two radial growth phase (RGP) melanoma cell lines, four vertical growth phase (VGP) melanoma cell lines, and seven metastatic melanoma cell lines (Figure 1b). Compared to THP-1 cells, several melanoma cell lines (WM1552C, WM793B, WM239A, A375, HS294T, and SK-MEL-2) had higher *NLRP1* RNA expression levels. Interestingly, we observed no clear correlation between *NLRP1* RNA expression (Figure 1b) and NLRP1 protein expression (Figure 1c) in these cell lines, nor any correlation between expression levels and melanoma growth phases (RGP, VGP or metastatic).

NLRP1 protein has been reported to be present in the nucleus of immune cells;[16] however, it is cytosolic NLRP1 protein that is thought to function as the driver of the NLRP1 inflammasome machinery.[16,24] To elucidate which compartment's NLRP1 was more relevant for human melanoma, we investigated the subcellular localization of NLRP1 in matched primary and metastatic melanoma cells (WM115/WM239A, WM278/WM1617, and WM793B/1205Lu) by Western blot analysis. Consistent with reported findings,[16] NLRP1 was predominantly expressed in the nucleus of THP-1 cells regardless of their differentiation by phorbol 12-myristate 13-acetate (PMA) and further activation of NLRP1 inflammasome by anthrax lethal toxin (LT)[25] (Figure 1d). In contrast, NLRP1 was principally expressed in the cytoplasm of melanoma cells (Figure 1e). No obvious differences in the subcellular distribution patterns of NLRP1 between primary and metastatic melanoma cells were observed. Immunofluorescence microscopy analysis revealed that NLRP1 is primarily in the nucleolus of THP-1 cells (Figure 1f), the dark region seen with 4′,6-diamidino-2-phenylindole (DAPI) staining of the nucleus,[26] whereas it is particularly abundant in the perinuclear region of cytoplasm in human melanoma cells. In accordance with previous reports,[16] NLRP3 was predominantly cytosolic in both THP-1 and melanoma cells. These data suggest that the differences in the subcellular localization of NLRP1 may reflect different biological roles in melanoma cells versus immune cells.

NLRP1 is a tumor promoter in human melanoma

To investigate the potential functional roles of NLRP1 in melanoma, we knocked down NLRP1 expression by transducing *NLRP1* shRNA into primary melanoma cells, WM35 and WM115, and metastatic melanoma cells, 1205Lu and HS294T. Successful transduction was confirmed by significant decreases in *NLRP1* RNA (Figure 2a) and NLRP1 protein (Figure 2b) compared to corresponding cells transduced with control shRNA. We first determined the effect of NLRP1 knockdown on viable cell numbers *in vitro*. Knocking down NLRP1 significantly reduced the viable cell numbers of WM115, 1205Lu, and HS294T cells, but not WM35 (Figure 2c).

To evaluate whether knocking down NLRP1 results in the same suppressive effects *in vivo*, we injected nude mice with *NLRP1* or control shRNA-transduced WM35 or 1205Lu cells and monitored tumorigenesis for 30 days by measuring tumor volumes at 2–3 day intervals. Mice injected with 1205Lu-*NLRP1*-shRNA cells showed significantly slower tumor growth and decreased tumor volume (63% reduction by day 25), compared to mice injected with 1205Lu-control-shRNA cells (Figure 2d). However, we were unable to evaluate the effect of NLRP1 knockdown in primary WM35 cells *in vivo* because WM35 cells rarely formed tumors even at the termination of the study (day 30).[27]

To confirm that NLRP1 promotes tumor growth in metastatic melanoma, additional animal experiments were performed (Supplementary Figure 1). In a second, independent animal experiment, we generated a new pool of shRNA-transduced cells by transducing the same shRNA into 1205Lu, and injected nude mice with newly generated 1205Lu-control-shRNA or 1205Lu-*NLRP1*-shRNA cells (Supplementary Figure 1a). To demonstrate that the observed NLRP1 knockdown effect is indeed on-target, we transfected 1205Lu cells with a *NLRP1* shRNA with non-overlapping sequences (obtained from a different company) and repeated animal experiments (Supplementary Figure 1b). To demonstrate that the tumor-promoting property of NLRP1 is not limited to 1205Lu, we injected nude mice with HS294T transduced with control or *NLRP1* shRNA plasmid (Supplementary Figure 1c). Taken together, these findings strongly indicate a role for NLRP1 in promoting tumor growth in metastatic melanoma.

NLRP1 inflammasomes are active in metastatic melanoma

IL-1β plays a critical role in tumor progression, immunosuppression, and chemoresistance. IL-1β is synthesized as a biologically inactive precursor and cleaved to biologically active mature IL-1β by caspase-1 whose activation is regulated by NLRP inflammasomes.[9,10] Spontaneous NLRP3 inflammasome activation and subsequent caspase-1 activation have been linked to IL-1β-mediated tumorigenesis in human melanoma.[8,28–30] However, it was unclear whether NLRP1 inflammasomes also mediate IL-1β activation in melanoma. We therefore investigated the participation of NLRP1 inflammasomes in the IL-1β activation and secretion pathways in human melanoma cells. Caspase-1 activation was evaluated by detecting the conversion of inactive pro-caspase-1 to an enzymatically active fragment of caspase-1 (p20) using Western blot analysis. In addition, we evaluated caspase-1 activity using fluorescent labeled inhibitors of caspases in WM35, WM115, 1205Lu, and HS294T cells. Consistent with our previously published data,[8] caspase-1 was constitutively cleaved

(indicated by the presence of 20 kDa fragment) in both primary and metastatic melanoma cells without exogenous stimuli (Figure 3a). Knockdown of NLRP1 significantly suppressed caspase-1 cleavage (Figure 3a) and caspase-1 activity (Figure 3b) in metastatic melanoma cells 1205Lu and HS294T but not in primary melanoma cells WM35 and WM115, suggesting that NLRP1 inflammasomes activate caspase-1 in metastatic melanoma.

Active caspase-1 cleaves pro-IL-1β, and active IL-1β is released into extracellular space, where it binds to IL-1 receptors and initiates inflammatory response by stimulating NF-κB activity.[11] In agreement with the effects of NLRP1 knockdown on caspase-1 activation in metastatic melanoma cells, knocking down NLRP1 significantly reduced IL-1β production (Figure 3c), IL-1β secretion (Figure 3d), and NF-kB activity (Figure 3e) in 1205Lu and HS294T cells. To evaluate the functionality of NLRP1 inflammasome in primary melanoma, NLRP1 was knocked down in WM35 and WM115 cells. Knocking down NLRP1 reduced IL-1β secretion in WM115 (Figure 3d), but this inhibitory effect was minimal due to the low levels of IL-1β production and secretion in primary melanoma cells. Different from the suppressive effects of NLRP1 on IL-1β production and NF-kB activity in metastatic melanoma cells, knocking down NLRP1 in primary melanoma cells did not reduce IL-1β production or NF-kB activity, but rather enhanced NF-kB activity. To examine the effect of functional NLRP1 inflammasome on NF-κB activity, we analyzed PMA-differentiated THP-1 cells. Knocking down NLRP1 significantly reduced NF-κB activity in THP-1 cells (Figures 3f–3g), but this effect was not due to the change in NLRP3 expression (Figure 3f), suggesting a positive regulation of NF-κB activity by NLRP1 in THP-1 cells. Taken together, these findings indicate that NLRP1 inflammasomes are functional and mediate IL-1β processing in metastatic melanoma.

NLRP1 knockdown does not affect the cell cycle but induces apoptosis in human melanoma cells

We have previously shown that ASC knockdown induces G1 cell cycle arrest, reduces viable cell numbers, and suppresses tumorigenesis in metastatic melanoma.[11] Because NLRP1 knockdown reduced viable cell numbers and suppressed tumorigenesis in metastatic melanoma, we examined whether NLRP1 knockdown also resulted in G1 cell cycle arrest. We analyzed cell proliferation by quantitating the proportion of cells residing in each phase of the cell cycle. Unlike ASC knockdown, NLRP1 knockdown did not alter the cell cycle ratios of 1205Lu and HS294T cells (Figure 4a), suggesting that NLRP1-mediated changes in cell viability and tumorigenesis are not related to cell proliferation. To investigate the mechanisms underlying the observed effects of NLRP1 on cell viability and tumorigenesis in human melanoma, we analyzed 1205Lu tumor tissues collected in the animal study depicted in Figure 2d. H&E staining revealed pyknotic cells in sections from 1205Lu-*NLRP1*-shRNA tumors (Figure 4b). Immunohistochemical analyses revealed slightly decreased proliferation (Ki-67-positive cells) but significantly increased apoptosis (TUNEL-positive cells) in tumors from mice injected with 1205Lu-*NLRP1*-shRNA cells compared with those from mice injected with 1205Lu-control-shRNA cells (Figure 4b).

We hypothesize that the CARD domain of NLRP1 may contribute to the regulation of apoptotic caspases resulting in the increase of apoptosis we observed *in vivo*. To this end, we

investigated cell apoptosis *in vitro* using Annexin V staining. NLRP1 knockdown increased apoptosis in 1205Lu cells (Figure 4c, Supplementary Figure 2). When cells were exposed to apoptosis inducer actinomycin D (AMD) for 18 h, NLRP1 knockdown increased apoptosis in both 1205Lu and HS294T cells. To evaluate whether the apoptosis-regulatory effects of NLRP1 is specific to metastatic melanoma cells, we analyzed THP-1 cells. Different from melanoma cells, knocking down NLRP1 did not result in an induction of apoptosis in AMD-treated THP-1 cells (Supplementary Figure 3). These data suggest that NLRP1 plays a role in the regulation of apoptosis in human melanoma cells.

NLRP1 protein interacts with caspase-2 and -9 and reduces caspase-3/7 activity in human melanoma cells

To further understand the molecular mechanisms by which NLRP1 controls apoptosis, we investigated the interaction of NLRP1 with CARD-containing apoptotic caspases in metastatic melanoma cells. Among 7 apoptotic caspases, only caspase-2 and -9 contain a CARD domain. Both caspases are initiator caspases for the intrinsic mitochondrial pathway leading to the processing of executioner caspases-3/7 that subsequently mediate the apoptotic cascade.[31] Co-immunoprecipitation analyses of endogenous proteins confirmed the interaction of NLRP1 with caspase-2 and -9 in 1205Lu and HS294T cells (Figure 5a, Supplementary Figure 4). NLRP3, which lacks a CARD motif, did not interact with caspase-2 and -9.

Next, we examined the effect of NLRP1 knockdown on caspase-2, -9, and -3/7 activities in 1205Lu and HS294T cells. Cells were cultured in the absence or presence of an apoptosis inducer, either AMD or camptothecin (CPT), which have been shown to induce activation of caspase-2, -9, and -3/7 (Figures 5b–5d). Knocking down NLRP1 led to increased caspase-2 (Figure 5b), -9 (Figure 5c), and -3/7 (Figure 5d) activities in untreated and apoptosis inducer-treated 1205Lu and HS294T cells, though the effects of NLRP1 knockdown on caspase-2 activity were relatively weak compared with those on caspase-9 and -3/7 activities. Knocking down NLRP1 also increased caspase-3/7 activity in WM35 and WM115 cells (Supplementary Figure 5).

To evaluate whether the caspase-suppressive effects of NLRP1 are specific to apoptotic caspases, we analyzed inflammatory caspase-1 activity in 1205Lu and HS294T cells (Supplementary Figure 6). In contrast to its effects on apoptotic caspases, knocking down NLRP1 reduced caspase-1 activity in the baseline (Figure 3b) and AMD-treated melanoma cells. These findings support our hypothesis that NLRP1 negatively regulates activation of the intrinsic apoptotic pathway in melanoma, irrespective of melanoma stages, and support the viability-promoting activity observed in Figure 2c.

NLRP1 inflammasome activation reduces caspase-2, -9 and -3/7 activity and apoptosis in human melanoma cells

Considering the role for NLRP1 polypeptide in apoptosis, we examined whether NLRP1 inflammasome activation also affects the intrinsic apoptotic pathway in melanoma cells. To examine the influence of differential inflammasome activation on caspase-2, -9, and -3/7 activities, NLRP1 inflammasomes were activated by LT whereas NLRP3 inflammasomes

were activated using the specific activator, monosodium urate (MSU).[9] Since inflammasome activation results in IL-1β secretion and subsequent activation of the IL-1 receptor, IL-1α was added to determine the effect of IL-1 receptor activation. After short-term treatment with LT, MSU or IL-1α, 1205Lu and HS294T cells were cultured in the absence or presence of AMD or CPT for 18 h. Activation of NLRP1 inflammasomes by LT significantly decreased AMD- or CPT-induced caspase-2 (Figure 6a), -9 (Figure 6b), and -3/7 (Figure 6c) activities. In contrast, NLRP3 inflammasome activation and IL-1α treatment decreased AMD-induced caspase-3/7 activity, but did not alter caspase-2 and -9 activities in 1205Lu and HS294T cells. Consistent with these results, flow cytometric analysis of apoptosis by Annexin V staining showed a reduction in apoptosis by LT in human melanoma cells (Figure 6d, Supplementary Figure 7).

Different from melanoma cells, activation of NLRP1 inflammasomes by LT did not reduce AMD- or CPT-induced caspase-2, -9 and -3/7 activities (Figures 6a–6c) or apoptosis (Figure 6d) in THP-1 cells, but rather increased caspase-2, -9 and -3/7 activities. To evaluate whether the caspase-suppressive effects of NLRP1 inflammsome are specific to apoptotic caspases, we analyzed inflammatory caspase-1 activity in 1205Lu and THP-1 cells (Supplementary Figure 8). In contrast to its suppressive effects on apoptotic caspases, NLRP1 inflammasome activation did not reduce caspase-1 activity in 1205Lu cells or THP-1 cells, but rather increased caspase-1 activity in THP-1 cells. Activation of the NLRP1 inflammasome by LT has been reported to induce caspase-1-dependent cell death, termed as pyroptosis, in macrophages of certain mouse species.[32–35] We therefore determined lactate dehydrogenase (LDH) release (indicative of pyroptosis)[36] in THP-1 and 1205Lu cells exposed to LT for 18 h. We found that LT increased LDH release in THP-1 cells, but not in 1205Lu cells (Figure 6e). Taken together, these data strongly support our conclusion that activation of NLRP1 inflammasomes protects human melanoma cells from apoptotic cell death.

DISCUSSION

While this manuscript was under revision, Zhong, et.al.[17] described gain-of-function mutations within the LRR and PYD domain of NLRP1. It was reported that while wild-type NLRP1 exists as a monomer in its inactive state, germline mutations in the PYD domain of NLRP1 lead to constitutively active NLRP1 which self-oligomerizes, activates the inflammasome, and promotes the processing of IL-1β. Patients with such mutations in *NLRP1* can suffer from skin disorders such as multiple healing palmoplantar carcinoma and familial keratosis lichenoides chronica.[17] These findings provide important genetic evidence linking inflammasome signaling to inflammatory skin disorders and skin cancer. Our findings further demonstrate that NLRP1 has at least two distinct biological functions: first, as a NLRP1 inflammasome to activate inflammatory caspase-1 and IL-1β, and second, as a NLRP1 polypeptide to interact with other CARD-containing proteins (particularly apoptotic caspases) that fine-tunes apoptosis. The former inflammasome function is active only in metastatic melanoma whereas the latter anti-apoptotic function is active in both primary and metastatic melanoma.

NLRP1 is important for eliciting the inflammatory response during host defense through the formation of inflammasome complex.[9,37] Concomitant with inflammatory response of

caspase-1 activation is pyroptotic cell death in macrophages of some mouse species.[32–35] Although we detected increased LDH release in LT-stimulated THP-1 cells, we did not detect this response in 1205Lu cells (Figure 6e). Instead, exposure of 1205Lu and HS294T melanoma cells to LT protected these cells from apoptotic cell death (Figures 6a–6d). NLRP1 contains both N-terminal pyrin domain and C-terminal CARD domain, and functions through the domain-domain interaction with inflammasome components or apoptosis signaling molecules. Caspase -2 and -9 are cytoplasmic proteins (Supplementary Figure 9), and the binding of NLRP1 to caspase-2 and -9 in melanoma cells may disrupt the interaction of these apoptotic caspases with other apoptosis-inducing proteins, thereby negatively regulating apoptosis in melanoma cells. In contrast, the nuclear distribution of NLRP1 in THP-1 cells may physically limit its interaction with caspase-2 and -9. This functional switch of NLRP1 from inducing cell death in non-melanoma models[38–40] to suppressing cell death in melanoma may represent an important mechanism contributing to tumor progression and metastasis.[41]

The anti-apoptotic function of NLRP1 may be of clinical importance in the treatment of cancer. Metastatic melanoma is notoriously resistant to chemotherapy. The apoptosis inducers AMD and CPT used in the present study are DNA intercalators and have a potent anti-tumor activity.[42,43] Such DNA-damaging agents interfere with DNA topoisomerases engaged in replication and transcription, subsequently activating the p53-mediated mitochondrial apoptotic pathway, in which caspase-2 and -9 act as key initiator caspases.[30,42–44] We observed more robust inhibitory effects of the NLRP1 inflammasome on activation of caspase-2 and -9 and apoptotic cell death in AMD- and CPT-treated cells than in untreated cells (Figures 5b–5d and Figures 6a–6d). NLRP1 also regulates activation of NF-κB (Figure 3e), which confers melanoma resistance to apoptosis.[45] Therefore, NLRP1 is not only an anti-apoptotic protein but also may mediate resistance to certain drugs, such as DNA-damaging agents.

Our results show that the NLRP1 inflammasome is involved in tumor-mediated inflammation and survival in metastatic melanoma, and that NLRP1 protein plays an anti-apoptotic role in melanoma. These two distinct properties of NLRP1 promote tumor growth and survival in human melanoma. This work begins to define the function of endogenous NLRP1 in melanoma, and further studies of NLRP1 and its regulators may further clarify its role in the molecular melanoma tumorigenesis and progression, which may reveal potential new therapeutic targets for human melanoma.

MATERIALS AND METHODS

Bioinformatics analysis

To examine NLRP1 expression in human melanoma tissues, we analyzed three online microarray profiling data sets: Study 1 (GEO accession number: GSE1506) from Raskin *et al.*[18] had 16 normal skin samples, 46 primary melanoma samples, and 12 metastatic melanoma samples, Study 2 (GSE7553) from Riker *et al.*[19] had 4, 14, and 40 samples, and Study 3 (GSE46517) from Kabbarah *et al.*[20] had 7, 31, and 73 samples, respectively. To re-normalize the Affymetrix array data, the intensity values of 3 probes per sample in each data set were averaged since they used the same internal control. For each data set, the average

expression level of *NLRP1* in normal skin samples was set as 1 and the expression of *NLRP1* in primary and metastatic melanoma tissues were adjusted accordingly.

Cell culture

THP-1 cells and 13 human melanoma cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 (Corning Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. THP-1 cells were differentiated with PMA (50 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA) overnight then treated with LT (List Biological Laboratories, Campbell, CA) containing 100 ng/ml lethal factor and 200 ng/ml protective antigen.

Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed using MMLV reverse transcriptase (Promega, Madison, WI). Quantitative real-time PCR was performed using the Power SYBR Green PCR Master Mix on the MX3000P PCR system (Applied Biosystems, Foster City, CA). The thermal profile was 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Primers for *NLRP1* were 5′-GCTGGACCAGACAACTCTGA-3′ (forward) and 5′-GGTTTCCGTCTGCTGAAGAT-3′ (reverse) and those for *GAPDH* were 5′-CAGGGCTGCTTTTAACTCTGG-3′ (forward) and 5′-TGGGTGGAATCATATTGGAACA-3′ (reverse).

Western blot

Cells were lysed in RIPA buffer containing 1% protease inhibitor cocktail (Sigma, St. Louis, MO). To assess the localization of NLRP1, cytoplasmic and nuclear fractions of cells were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL). Primary antibodies included NLRP1 (Enzo Life Sciences, Farmingdale, NY, #ALX-210-017 or ALX-804-803), HSP90 (ALX-804-078), NLRP3 (Cell Signaling Technology, Danvers, MA, #13158), caspase-1 (#2225), cyclophilin A (CyPA, #2175), and Lamin B (Santa Cruz Biotechnology, #sc-2617). Signals were visualized by SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and analyzed using the Odyssey imaging system (LI-COR, Lincoln, NE). The band densities were quantified using the ImageJ software (NIH, MD).

Confocal imaging

Cells were seeded on cover glasses. For THP-1 cells, PMA was added to induce differentiation. Cells were fixed in 4% paraformaldehyde and permeabilized by 0.25% Triton X-100. Following blocking, cells were serially incubated in rabbit anti-NLRP1 (1:400) and Alexa Fluor 488 goat anti-rabbit IgG (1:1000; Invitrogen). Cells were mounted with Prolong Gold anti-fade reagent with DAPI (Invitrogen) and imaged under an Olympus FV-1000 microscope (Olympus America, Center Valley, PA) with a 60X water lens.

shRNA transduction

Two different sets of *NLRP1* shRNAs were used for NLRP1 knockdown (sequences are shown in Supplementary Tables 1 and 2). Briefly, cells were transduced overnight with 1×10^5 infectious units of virus of shRNA lentiviral particles against control or *NLRP1* (Santa Cruz Biotechnology) in culture medium containing 5 μg/ml Polybrene (Santa Cruz Biotechnology) in 24-well plates. Cells expressing transduced shRNA were selected by and maintained in culture medium with 1 μg/ml of puromycin (Sigma). To confirm that the observed phenotype by knocking down NLRP1 is on-target, cells were also transfected with 250 ng of scrambled or *NLRP1* shRNA expression pGFP-V-RS vectors (Origene, Rockland, MA) using Lipofectamine 2000 (Invitrogen) for 24 h. GFP-positive cells were sorted on a MoFlo XDP100 sorter (Beckman, Indianapolis, IN).

siRNA transfection

Cells were transfected with 40 nM *NLRP1* siRNA (a mixture of two preselected siRNAs for *NLRP1*) or negative control (Qiagen Valencia, CA) using Lipofectamine 2000 reagent in OPTI-MEM1 reduced serum medium overnight.

Cell viability assay

Cell viability was measured as described previously using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega).[11]

Tumor formation *in vivo*

Six-week-old female athymic *nu*/*nu* mice from Jackson Laboratories (Bar Harbor, ME) were randomly assigned to two experimental groups and all experimental manipulations were approved by the Institutional Animal Care and Use Committee of University of Colorado Denver. In the first set of experiments, a total of 1×10^6 shRNA lentiviral particle-transduced 1205Lu cells or 2.5×10^6 shRNA lentiviral particle-transduced WM35 cells were suspended in 0.1 ml of Matrigel Matrix (BD Biosciences, San Jose, CA) diluted 1:1 with phosphate buffered saline and injected intradermally into the flank of mice (2 tumors per mouse, 4 mice per group). In the second animal study, which are an extension of the first set, 1205Lu (1.8×10^5) and HS294T (2×10^5 cells) cells carrying control or *NLRP1* shRNA plasmids were separately injected into nude mice (total 5 and 4 mice, 2 tumors each, for 1205Lu and HS294T, respectively). In the third animal study, 9×10^4 GFP-positive 1205Lu cells transfected with different control or *NLRP1* shRNA plasmids were injected per flank of each mouse of 3 mice per group. Tumor growth was monitored regularly with an digital caliper and tumor volume was calculated with the formula: tumor volume (mm^3) = longest diameter × shortest $diameter^2/2$.

Immunohistochemistry

Tumor tissues were harvested and fixed in 10% neutral buffered formalin. After paraffin embedding, tumor specimens were cut into 5-μm sections and stained with hematoxylin and eosin (H&E). Ki-67 staining and TdT-mediated dUTP nick end labeling (TUNEL) were performed by IHC World (Woodstock, MD) following its standard protocols.

ELISA

As described previously,[11] culture supernatants were collected for the analysis of secreted IL-1β and cell lysates collected for the analysis of IL-1β production (intracellular IL-1β) using a human IL-1β ELISA kit (R&D Systems, Minneapolis, MN).

NF-kB activity assay

Cells seeded in 24-well plates at 5×10^4 cells/well overnight were transfected with a control vector (pMetLuc2) or an NF-κB reporter vector (pNFkB-MetLuc2) (Clontech Laboratories, San Francisco, CA) using Lipofectamine 2000 (Invitrogen). Culture medium was collected 18 h later for the analysis of NF-κB activity using the Ready-to-Glow Secreted Luciferase Vector kit (Clontech Laboratories).

Cell cycle analysis

Cell cycle analysis was performed as described previously.[11]

Immunoprecipitation

Pre-cleared whole cell lysates were incubated with a primary antibody or control IgG then immunoprecipitated with TrueBlot anti-rabbit or anti-mouse Ig IP beads (Rockland Immunochemicals, Limerick, PA). Immunoprecipitates were analyzed by Western blot. TrueBlot secondary antibodies (Rockland Immunochemicals) were used to reduce interference by the ~55 kDa heavy and ~23 kDa light chains of the immunoprecipitating antibody.

Caspase activity assay

To determine the activity of the caspases of interest, cells were treated with DMSO as a vehicle control or an apoptosis inducer, AMD (5 μM) or CPT (1 μM) (BioVision, Milpitas, CA), for 18 h. To determine the effect of inflammasome activation on the caspase activities, cells were pre-treated with LT, MSU (50 μg/ml; Enzo Life Sciences), or recombinant human IL-1α (10 ng/ml; ThermoFisher Scientific) for 2 h prior to the addition of apoptosis inducers. Caspase-1 and -9 activities were measured using FAM-FLICA caspase assay kits (ImmunoChemistry Technologies, Bloomington, MN) with a fluorescence plate reader. Caspase-2 and -3/7 activities were measured using Caspase-Glo kits from Promega. To selectively measure caspase-2 activity, caspase-3/7 inhibitor Ac-DEVD-CHO (60 nM; Enzo Life Sciences) and proteasome inhibitor Z-Leu-leu-leu-al (60 μM; Sigma) were added to the Caspase-Glo 2 reagent as recommended by the Caspase-Glo 2 kit protocol.

Annexin V staining

Cells were stained with Annexin V and PI according to the FITC Annexin V apoptosis detection kit I protocol (BD Biosciences) for flow cytometric analysis. Early apoptotic cells (Annexin V-positive/PI-negative) and end stage apoptotic cells (Annexin V-positive/PI-positive) were quantitated.

LDH activity assay

LDH in culture medium was assayed using the Pierce LDH cytotoxicity assay kit (Thermo Scientific) according to the manufacturer's protocol.

Statistical analysis

Results represent at least three independent experiments. The numerical data are expressed as mean ± s.e.m. Two-tailed Student's *t*-test was used for two groups only, while for three or more groups, one-way ANOVA was performed with Bonferroni post-test using the GraphPad Software. $P < 0.05$ was considered statistically significant. Assumptions of normal distribution and similar variance were examined per statistical analysis when applicable. No samples were excluded from the data analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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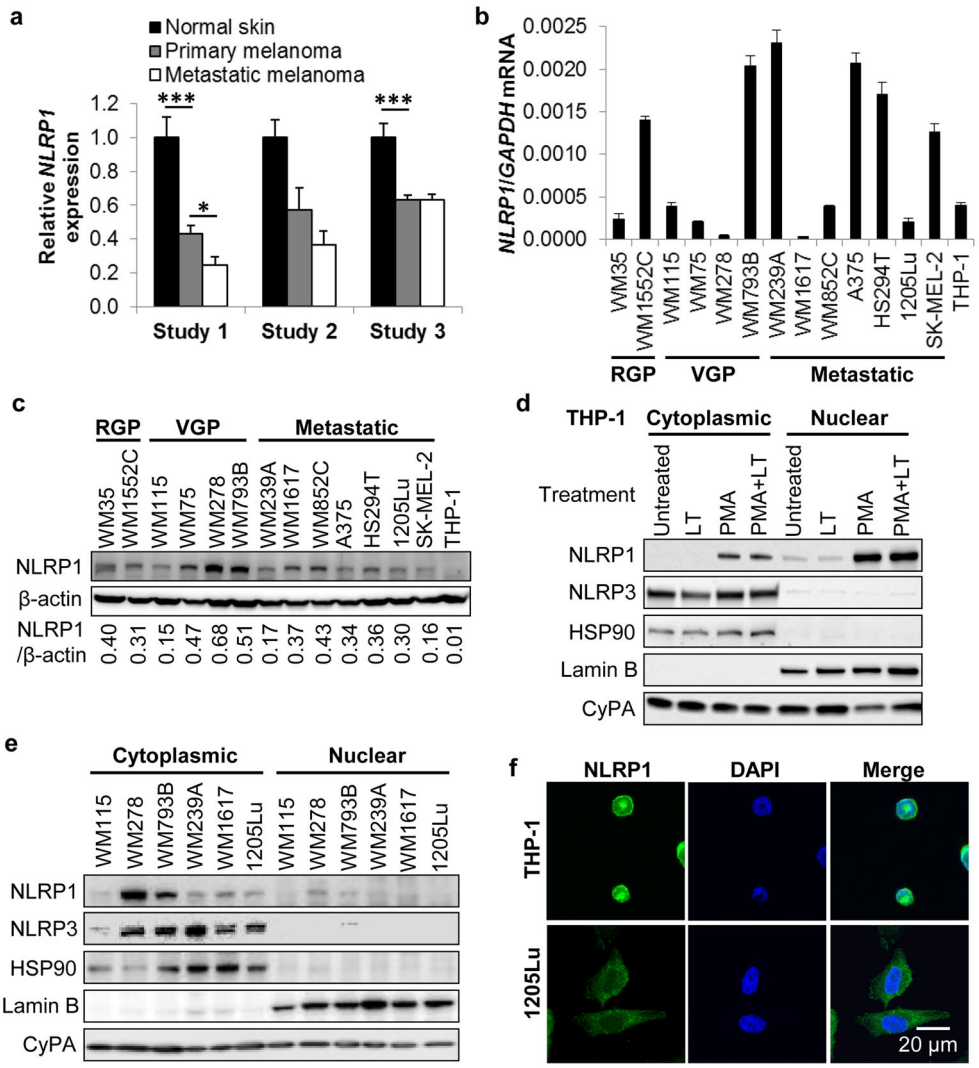

Figure 1. NLRP1 expression in human melanoma cells. (a) Microarray analyses of *NLRP1* RNA expression in human melanoma tissues

The data from three independent gene profiling studies were used to compare *NLRP1* RNA expression levels in human normal skin, primary melanoma and metastatic melanoma. The first study from Raskin *et al.*[18] had 16 normal skin samples, 46 primary melanoma samples, and 12 metastatic melanoma samples. The second study from Riker *et al.*[19] had 4 normal skin samples, 14 primary melanoma samples, and 40 metastatic melanoma samples, and the third study from Kabbarah *et al.*[20] had 7 normal skin samples, 31 primary melanoma samples, and 73 metastatic melanoma samples. See Materials and Methods for normalizing the affymetrix array data and statistical comparison. Data represent mean ± s.e.m. (**b**) Quantitative RT-PCR of *NLRP1* RNA in human melanoma cell lines and differentiated human monocytic THP-1 cells. RGP: radial-growth phase melanoma; VGP: vertical-growth phase melanoma. Data represent mean ± s.e.m. for triplicate experiments except for 1205Lu, HS294T, A375, and WM35 with sextuplicate experiments. (**c**) Western blot analyses of NLRP1 protein expression levels in human melanoma cell lines and differentiated THP-1 cells. The band intensities were quantitated and the ratios of NLRP1/β-actin calculated. (**d**)

Western blot analysis of intracellular localization of NLRP1 in THP-1 cells. THP-1 cells were untreated (undifferentiated), differentiated with phorbol 12-myristate 13-acetate (PMA) or further stimulated with anthrax lethal toxin (LT). Cytoplasmic and nuclear fractions of THP-1 cells were isolated and assayed for NLRP1 and NLRP3 expression. HSP90 and Lamin B were used as markers for cytoplasmic and nuclear proteins, respectively. Cyclophilin A (CyPA) is expressed in the cytoplasm and nucleus of all cell types. (**e**) Similar to (d), Western blot analysis of intracellular localization of NLRP1 and NLRP3 in matched primary and metastatic melanoma cells (WM115/WM239A, WM278/WM1617, and WM793B/1205Lu). Representative blots are shown. (**f**) Immunofluorescence staining of NLRP1 in human melanoma 1205Lu cells and monocytic THP-1. Cells were stained for NLRP1 and nucleus using Alexa Fluor 488 secondary antibody conjugated (green) and DAPI (blue), respectively. Representative staining cells of quadruplicate experiments are shown.

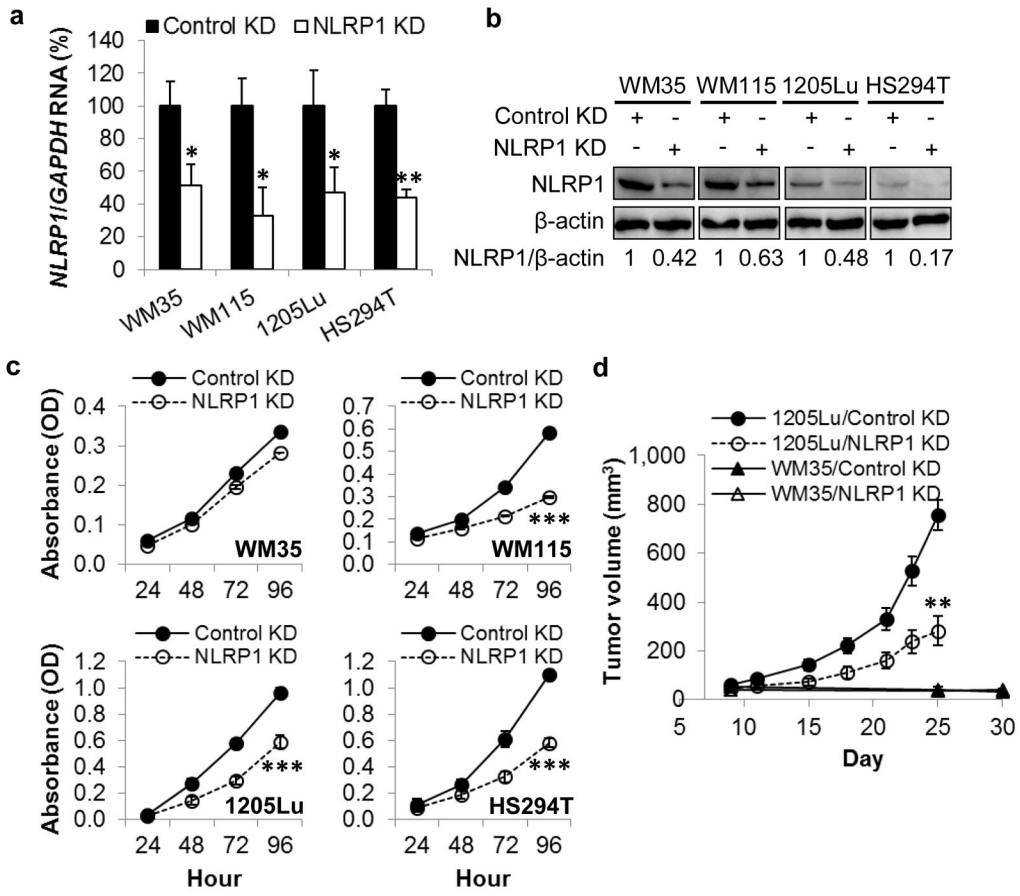

Figure 2. Effects of NLRP1 knockdown on human melanoma cell viability *in vitro* and tumor growth *in vivo*

(**a**) Real-time RT-PCR analysis of *NLRP1* RNA expression levels in human melanoma cells transduced with control or *NLRP1* shRNA. *NLRP1* gene expression levels in the control shRNA-expressing cells were normalized to 100%. Data represent mean ± s.e.m for triplicate experiments. (**b**) Western blot analysis of NLRP1 protein expression levels in melanoma cells with stable NLRP1 knockdown. Optical density of NLRP1 was quantitated and normalized to that of corresponding loading control β-actin. (**c**) Cell viability of melanoma cells with NLRP1 knockdown and corresponding control cells with mock transduction. Cell viability was determined daily for four days by the MTS-based assay. Data represent mean ± s.e.m. for triplicate (1205Lu and HS294T) or quadruplicate (WM35 and WM115) experiments. (**d**) Effects of NLRP1 knockdown on 1205Lu and WM35 tumor growth *in vivo*. Nude mice were injected with control shRNA- or *NLRP1* shRNA (Santa Cruz Biotechnology)-transduced WM35 cells (2.5×10^6) or 1205Lu cells (1×10^6), and tumor growth was monitored for 30 days. Data represent mean ± s.e.m. of eight tumors. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with corresponding cells with control shRNA-expression or tumor tissues. KD, knockdown. OD, optical density.

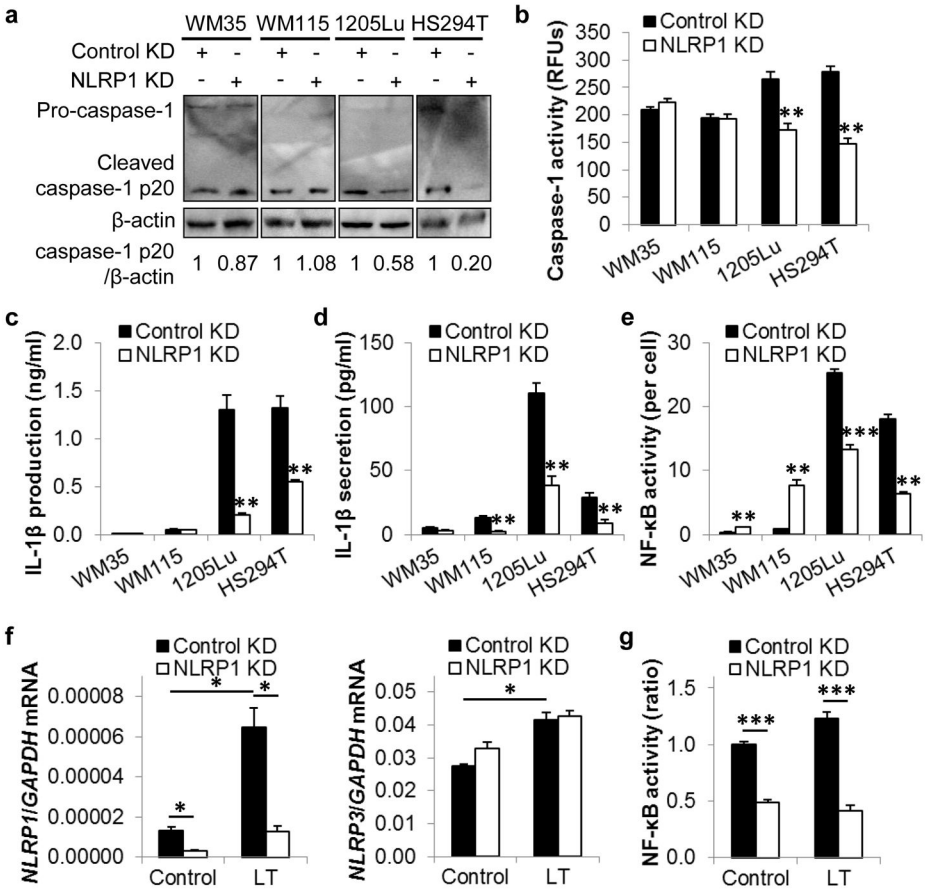

Figure 3. Effects of NLRP1 knockdown on NLRP1 inflammasome function and NF-κB activity in melanoma cells

(**a**) Western blot analysis of caspase-1 cleavage indicated by the presence of 20 kDa fragment in four melanoma cells. The band densities of cleaved caspase-1 p20 were quantitated and normalized to that of the corresponding loading control β-actin. (**b**) Caspase-1 activity after NLRP1 knockdown in human melanoma cells determined by a FAM-FLICA (fluorescent labeled inhibitors of caspases caspase) assay. (**c**) IL-1β production in human melanoma cells with NLRP1 knockdown. Cells were cultured in OPTI-MEM I reduced serum medium for 24 h. Cell lysates were assayed for IL-1β production by ELISA. (**d**) IL-1β secretion in human melanoma cells with NLRP1 knockdown. Similar to (c), however, culture medium was collected for assessing IL-1β secretion. (**e**) NF-κB activity in melanoma cells with NLRP1 knockdown determined by a Ready-to-Glow Secreted Luciferase assay. (**f**) *NLRP1* and *NLRP3* RNA expression in THP-1 cells transfected with *NLRP1* siRNA. THP-1 cells were differentiated with PMA then treated with LT for another 24 h. (**g**) NF-κB activity in THP-1 cells transfected with *NLRP1* siRNA. Data represent mean ± s.e.m. for triplicate experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with corresponding mock-transduced control cells. KD, knockdown.

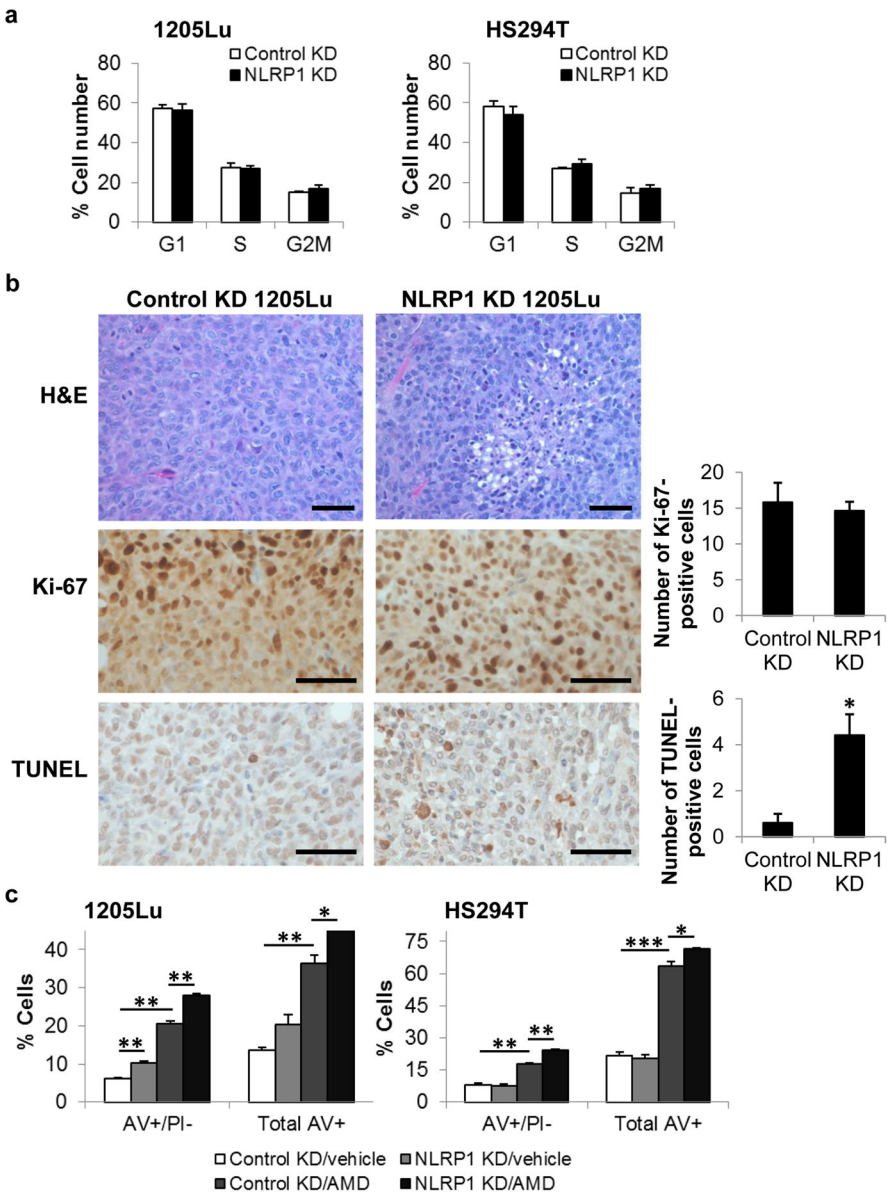

Figure 4. Effects of NLRP1 knockdown on cell proliferation and apoptosis in human melanoma cells

(**a**) Cell cycle analysis of 1205Lu and HS294T cells transduced with control or *NLRP1* shRNA. Data represent mean ± s.e.m. for triplicate experiments. (**b**) Representative 1205Lu tumor sections stained with hematoxylin and eosin (H&E) (upper panel), Ki-67 (middle panel), and for apoptosis using TUNEL method (lower panel). Tumor sections were prepared from Figure 2d tumor tissues. Bar = 50 μm. Quantitative analyses of Ki-67-positive cells and TUNEL-positive cells were performed with cell counting in the whole field under a microscope with 200 × magnification. Data represent mean ± s.e.m. for quadruplicate experiments. *$P < 0.05$ versus tumors from 1205Lu-control-shRNA cells. (**c**) Flow cytometric analysis of apoptosis with Annexin V conjugate. Cells were treated with DMSO (vehicle) or an apoptosis inducer actinomycin D (AMD, 5 μM) for 18 h. Data represent

mean ± s.e.m. for triplicate experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. KD, knockdown. AV, Annexin V.

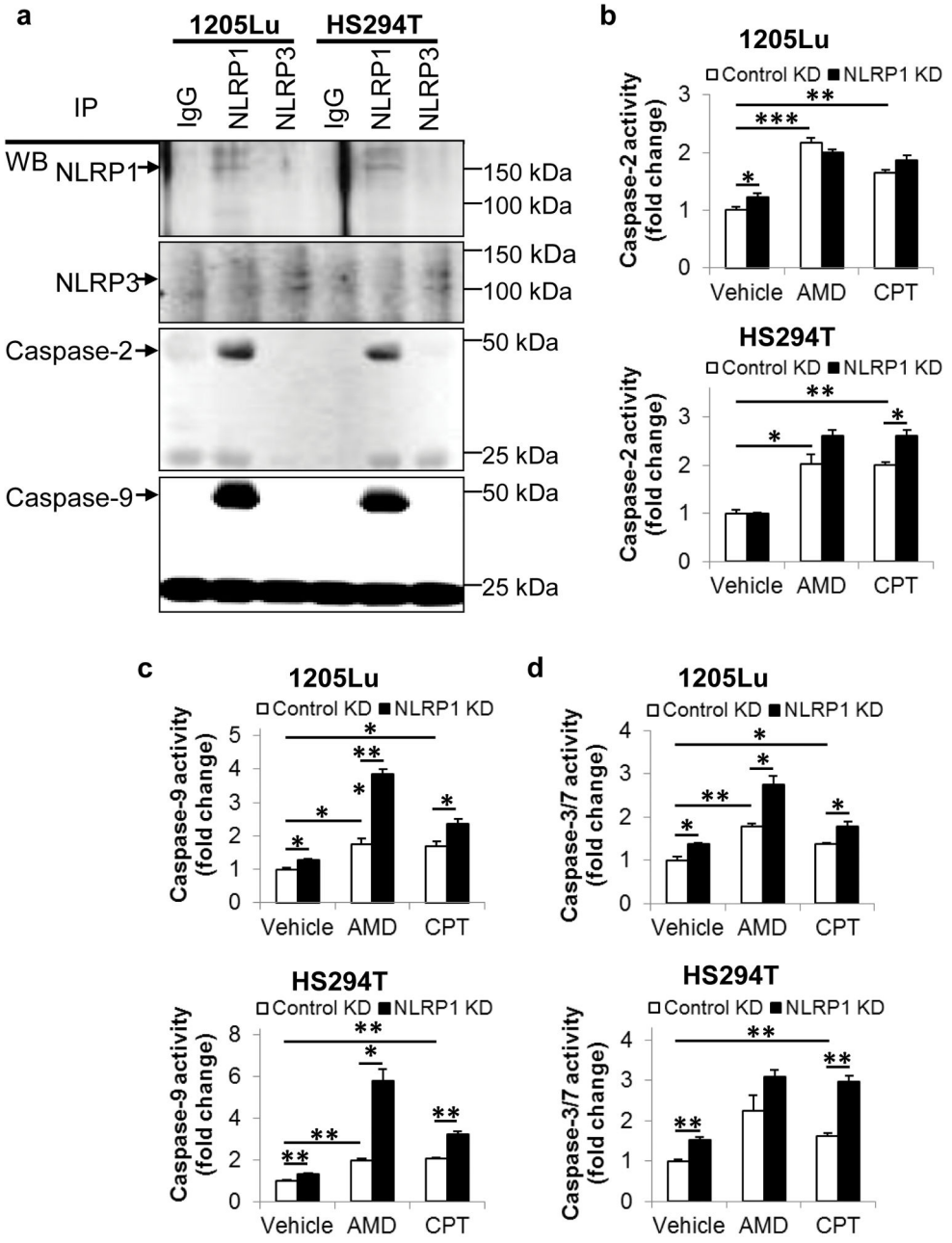

Figure 5. Effects of NLRP1 knockdown on pro-apoptotic caspase activities in human melanoma cells

(**a**) Immunoprecipitation interaction of NLRP1 with caspase-2 and caspase-9 in human melanoma cells. Whole lysates of 1205Lu and HS294T cells were immunoprecipitated with IgG control (IgG), anti-NLRP1 (NLRP1) or anti-NLRP3 (NLRP3) antibody, subjected to SDS-PAGE and probed with antibodies against NLRP1, NLRP3, caspase-2, and caspase-9 for Western blot (WB). (**b, c, d**) Caspase-2 (**b**), caspase-9 (**c**), and caspase-3/7 (**d**) activities in *NLRP1* shRNA-expressing 1205Lu and HS294T cells treated with DMSO (vehicle), 5 μM actinomycin D (AMD) or 1 μM camptothecin (CPT) for 18 h. Data represent mean ±

s.e.m. for triplicate experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. IP, immunoprecipitation. KD, knockdown.

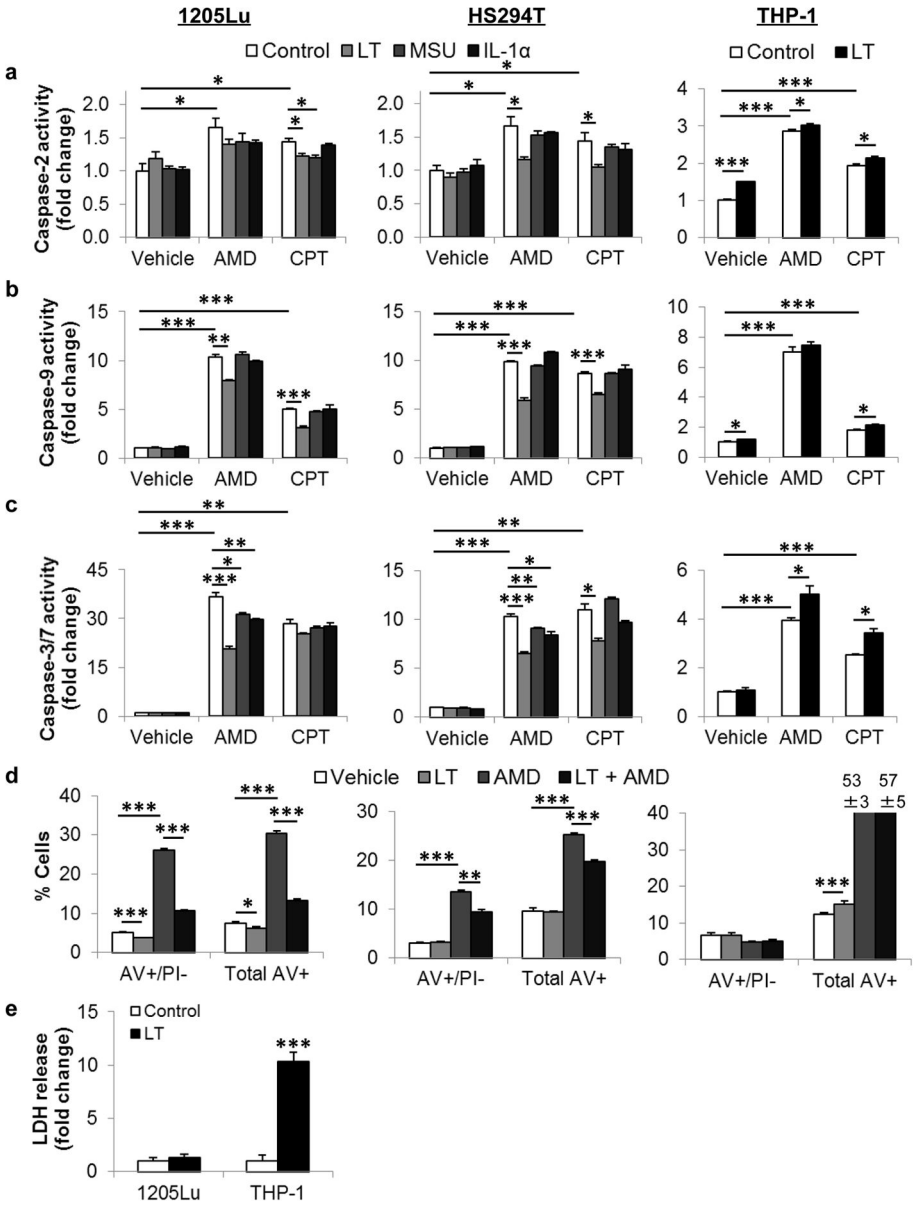

Figure 6. Effects of NLRP1 inflammasome activation on pro-apoptotic caspase activities and apoptosis in human melanoma cells and THP-1 cells

(**a, b, c**) Caspase-2 (**a**), caspase-9 (**b**), and caspase-3/7 (**c**) activities in 1205Lu, HS294T, and THP-1 cells, respectively. As indicated, cells were stimulated with NLRP1 inflammasome activator LT containing 100 ng/ml lethal factor and 200 ng/ml protective antigen, NLRP3 inflammasome activator monosodium urate (MSU, 50 μg/ml), or recombinant human IL-1α (10 ng/ml) for 2 h, and subsequently treated with AMD or CPT for another 18 h. (**d**) Flow cytometric analysis of apoptosis with Annexin V staining in 1205Lu, HS294T, and THP-1 cells. Cells were pretreated with LT for 2 h and treated with AMD for 18 h. (**e**) Lactate dehydrogenase (LDH) release by 1205Lu and THP-1 cells after exposure to LT for 18 h. Data represent mean ± s.e.m. for triplicate (caspase-2 activity and Annexin V staining) or

quadruplicate (caspase-9 activity, caspase-3/7 activity, and LDH release) experiments. *$P <$ 0.05, **$P < 0.01$, and ***$P < 0.001$. KD, knockdown. AV, Annexin V.